Attorney Docket No. 125192.00501

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		:	
Hiroharu Kawahara		:	Confirmation No.: 1684
Serial No.:	10/591,521	:	Group Art Unit: 1656
Filed:	September 1, 2006	:	Examiner: Kim, Alexander D.

Title: HUMAN CELL STRAINS FOR PROTEIN PRODUCTION, PROVIDED BY SELECTING STRAINS WITH HIGH INTRACELLULAR PROTEIN AND MUTATING WITH CARCINOGENS

DECLARATION OF HIROHARU KAWAHARA

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Commissioner for Patents
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1. I, Hiroharu Kawahara, hereby declare as follows:

2. Under my direct supervision, the following two experiments were conducted.

3. **Experiment 1:**
A recombinant gene vector was transfected into each of the cell strains: SC-01MFP, RPMI8226, SC-02MFP, and KMS-12BM, and subsequently each cell stain was cultured under the same incubation condition.

4. **Material and Method**
The cell concentrations of the four cell strains: SC-01MFP, RPMI8226, SC-02MFP, and KMS-12BM, were each prepared to 1x10^7 cells/ml with a phosphate buffered saline (PBS). 500µl of each cell suspension was added to a sample tube, and 1µl (final concentration of 1µg/ml) of a recombinant gene vector containing a cytomegalovirus promoter, a G418 drug resistant gene, and a gene encoding human antibody heavy chain was added. This suspension was transferred to 0.4 cm cuvettes for a Gene Pulsar, transgenic device (using in vivo electroporation). The cuvettes were inserted into the electrode of the Gene Pulsar, the voltage was set at 0.4 kV (1.0kV/cm), then applied at 300µF. Next, it was transferred to a RPMI1640 medium, and let stand for 5 minutes in a centrifuge tube. Then this centrifuge tube was centrifuged for 5 minutes at 400 x g. The centrifuged supernatant was discarded, and after being suspended with 5 ml of 15% FBS-RPMI medium, it was dispensed into a 96 well culture plate at 100µl/well. 2 – 4 days later, GENETICIN;antibiotic G-418

sulfate (final concentration of 2μg/ml) was added, and selective culturing, where the cells other than the gene-transfected cells were terminated, was carried out. For several weeks, the medium was changed continuously with fresh medium containing GENETICIN.

5. **Results**

Since cell proliferation was confirmed in several weeks (2-4 weeks), the protein weight of this antibody heavy chain (γ chain) was measured using an enzyme antibody technique. The SC-01MFP and SC-02MFP cell strains maintain a stable protein production over 2 months period. As for the RPMI8226 cell strain and the KMS-12BM cell strain, after the transfection of the gene into the RPMI8226 cell strain and the KMS-12BM cell strain, the antibody heavy chain protein from the transfected gene were temporally synthesized, but by the 30th day after the transfection the production fell below the detection limit of γ chain protein by the enzyme antibody technique, thus the production of the protein from the transfected gene disappeared . Fig. 1 shows the results.

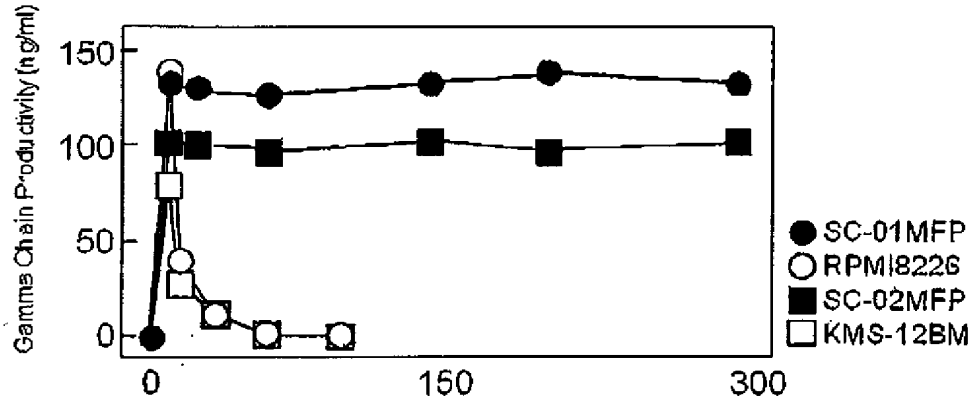

Fig.1 Productivity of Antibody Heavy Chain Protein

6. **Experiment 2:**
Four cell strains, SC-01MFP, RPMI8226, SC-02MFP, and KMS-12BM were incubated in a serum free medium.

7. **Material and Method**
The cell concentration of each cell strain was prepared to 1x10^5 cells/ml.
Next, it was incubated in a medium called ITES-ERDF, where 10μg/ml of insulin (I), 20μg/ml of transferring (T), 20μM of ethanolamine (E), and 25nM of sodium selenite (S) are added to a minimal essential medium ERDF (Kyokuto Pharmaceutical) as final concentration.

8. **Results**
RPMI8226 and KMS-12BM did not proliferate as the result of incubating in the ITES-ERDF medium, while SC-01MFP and SC-02MFP proliferated. Fig. 2 shows the results.

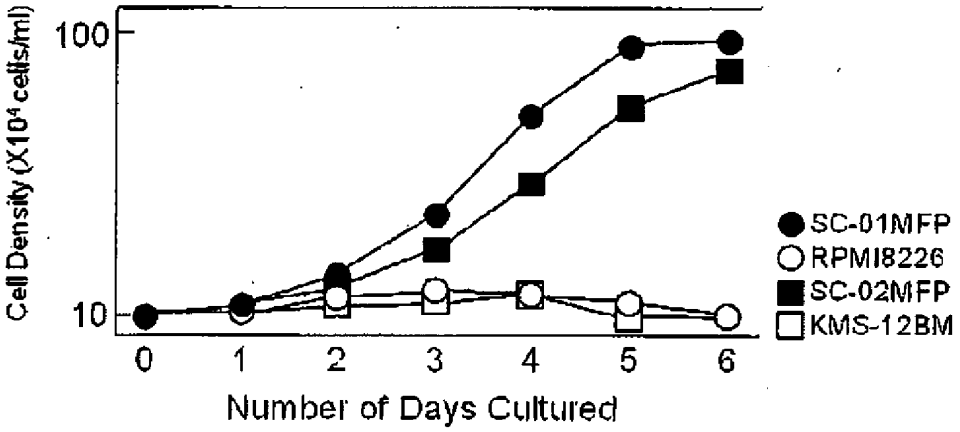

Fig. 2 Proliferation when incubated in a serum free culture

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

Respectfully submitted,

Dated: 03-Mar-'09

By: _____

Hiroharu Kawahara